Modulation of NKG2D, NKp46, and Ly49C/I facilitates natural killer cell-mediated control of lung cancer

Lei Shi[a,b,c,1], Kang Li[a,b,c,1], Yizhan Guo[b,c], Anirban Banerjee[b,c], Qing Wang[b,c], Ulrike M. Lorenz[c,d], Mahmut Parlak[c,d], Lucy C. Sullivan[e], Oscar Okwudiri Onyema[b,c], Saeed Arefanian[f], Edward B. Stelow[g], David L. Brautigan[g], Timothy N. J. Bullock[c,g], Michael G. Brown[c,h], and Alexander Sasha Krupnick[b,c,2]

[a]The First Affiliated Hospital of Xi'an Jiaotong University, Shaanxi 710049, China; [b]Department of Surgery, University of Virginia, Charlottesville, VA 22908; [c]Carter Immunology Center, University of Virginia, Charlottesville, VA 22908; [d]Department of Microbiology, Immunology, and Cancer Biology, University of Virginia, Charlottesville, VA 22908; [e]Department of Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Melbourne, VIC 3000, Australia; [f]Department of Surgery, Washington University, St. Louis, MO 43110; [g]Department of Pathology, University of Virginia, Charlottesville, VA 22908; and [h]Department of Medicine, Division of Nephrology, University of Virginia, Charlottesville, VA 22908

Edited by Lewis L. Lanier, University of California, San Francisco, CA, and approved October 5, 2018 (received for review March 21, 2018)

Natural killer (NK) cells play a critical role in controlling malignancies. Susceptibility or resistance to lung cancer, for example, specifically depends on NK cell function. Nevertheless, intrinsic factors that control NK cell-mediated clearance of lung cancer are unknown. Here we report that NK cells exposed to exogenous major histocompatibility class I (MHCI) provide a significant immunologic barrier to the growth and progression of malignancy. Clearance of lung cancer is facilitated by up-regulation of NKG2D, NKp46, and other activating receptors upon exposure to environmental MHCI. Surface expression of the inhibitory receptor Ly49C/I, on the other hand, is down-regulated upon exposure to tumor-bearing tissue. We thus demonstrate that NK cells exhibit dynamic plasticity in surface expression of both activating and inhibitory receptors based on the environmental context. Our data suggest that altering the activation state of NK cells may contribute to immunologic control of lung and possibly other cancers.

NK cells | lung cancer | immunosurveillance | tumor immunology | immunotherapy

While natural killer (NK) cells play an important role in controlling hematologic malignancies (1), they also present a major immunologic barrier to the development of certain solid tumors, such as lung cancer (2). We and others have demonstrated that robust or weak NK cell function correlates with resistance or susceptibility to lung cancer, respectively (2–4). NK cells recognize targets through surface-activating receptors that detect stress ligands expressed by transformed or stressed cells. Stress ligands recognized by NKG2D- and NKp46 (NCR1)-activating receptors, for example, have been reported to control the growth and metastasis of lung cancer (2, 5). Concurrent inhibitory ligand expression on targets can block activating receptor-mediated NK cell cytotoxicity, a process known as "target interference" (6). Such inhibition occurs through target expression of major histocompatibility class I (MHCI) and other surface ligands that engage a variety of inhibitory receptors (7). Since malignancies can express MHCI, the balance in strength of activating and inhibitory receptors may control tumor destruction or escape.

Environmental MHCI plays another critical role in NK cell function by determining their responsiveness to stimulation. NK cells developing in an MHCI-deficient ($MHCI^{-/-}$) environment, or in the absence of MHCI-specific inhibitory receptors, display a global hyporesponsiveness and lyse MHCI-deficient targets with low avidity (8, 9). Responsiveness can be altered even in mature NK cells upon re-exposure to MHCI (10–13). Lung cancer presents a unique MHCI-positive solid tumor, the clinical course of which is heavily determined by NK cell reactivity. Despite its clinical importance, mechanistic aspects of NK cell physiology that control lung cancer growth are poorly defined.

Here we demonstrate that MHCI exposure facilitates clearance of lung cancer by increasing the levels of several key activating receptors and downstream signaling intermediates important for signal transduction by NK cells. We further show that under certain conditions NK cells can down-regulate the surface expression of the Ly49C/I inhibitory receptor, thus eliminating target interferences by MHCI-positive tumors. Our data describe an unusual plasticity of NK cells and demonstrate that this cell population may dynamically adjust to environmental stimuli to optimize control of pathogens.

Results

MHC Class I Expression Contributes to NK Cell-Mediated Control of Lung Cancer. We and others have previously shown that NK cell cytotoxicity directly contributes to susceptibility or resistance to lung cancer (2, 3). Thus, to evaluate the role of MHCI-mediated NK cell licensing or education in lung cancer growth, we injected Lewis Lung Carcinoma (LLC) into mice rendered $MHCI^{-/-}$ due to deletion of β2-microglobulin in parallel to MHC class I-positive ($MHCI^{+/+}$) control mice. As $CD8^+$ T cells fail to normally develop in $MHCI^{-/-}$ animals, we included $CD8^+$ T cell-deficient mice as $MHCI^{+/+}$ controls (14, 15). This allowed us to evaluate tumor growth in a model system where NK cells are the dominant cytotoxic effector cells due to either developmental or genetic deletion of $CD8^+$

Significance

Lung cancer is unique among solid tumors as robust natural killer (NK) cell function correlates with resistance to disease. Here we describe that NK cell education by major histocompatibility class I (MHCI) leads to the up-regulation of NKG2D- and NKp46-activating receptors that recognize lung cancer. We further demonstrate that upon activation NK cells down-regulate the expression of the Ly49C/I inhibitory receptor, thus eliminating target interference by MHCI. Our findings are significant based on the demonstration that NK cells that arise in an $MHCI^{+/+}$ environment present a substantial barrier to the growth of lung cancer, and expression of both activating and inhibitory receptors is not fixed but varies based on environmental context.

Author contributions: L.S., K.L., A.B., D.L.B., T.N.J.B., M.G.B., and A.S.K. designed research; L.S., K.L., Y.G., A.B., Q.W., U.M.L., M.P., O.O.O., S.A., E.B.S., and A.S.K. performed research; U.M.L. and L.C.S. contributed new reagents/analytic tools; L.S., K.L., Y.G., A.B., Q.W., U.M.L., O.O.O., E.B.S., D.L.B., T.N.J.B., M.G.B., and A.S.K. analyzed data; and L.S., K.L., A.B., and A.S.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: Data reported in this paper have been deposited in FigShare at https://figshare.com/s/a4d994e18c0c413f318c.

[1]L.S. and K.L. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: sashak@virginia.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1804931115/-/DCSupplemental.

Published online October 31, 2018.

T cells. Minimal LLC tumor growth was evident in MHCI$^{+/+}$ mice, with functionally competent NK cells. The lungs of MHCI$^{-/-}$ mice, in contrast, were mostly replaced with LLC tumors by day 16 post-injection (Fig. 1*A*). Similar infiltration of myeloid cells was evident in MHCI$^{+/+}$ and MHCI$^{-/-}$ animals (*SI Appendix*, Fig. S1*A*) but differences in antitumor activity were fully ablated when NK cells were depleted before injection of LLC (Fig. 1*A*). Similar differences in tumor growth were observed after s.c. injection of LLC as well (*SI Appendix*, Fig. S1*B*). Furthermore, freshly isolated splenic NK cells from MHCI$^{+/+}$ mice readily lysed LLC targets while NK cells from MHCI$^{-/-}$ animals demonstrated little cytotoxicity (Fig. 1*B*). While such in vitro and in vivo differences in NK cell biology have been previously described for MHCI$^{-/-}$ targets (8), LLC expresses MHCI at levels similar to other nonhematopoietic cells in the tumor microenvironment (Fig. 1*C*). Thus, NK cell licensing, or education, also contributes to control of MHCI$^{+/+}$ malignancies.

In addition to transplantation of LLC tumor cells, we injected MHCI$^{-/-}$ and MHCI$^{+/+}$ mice with the lung carcinogen ethyl carbamate and quantitated lung cancer by necropsy 6 mo later. Similar to LLC, lung cancer induced by primary carcinogenesis grew robustly in MHCI$^{-/-}$ animals (Fig. 1*D*). Similar differences were evident in other NK cell-responsive tumors such as fibrosarcoma (16) (*SI Appendix*, Fig. S1*C*). Taken together, our results suggest that host MHCI expression may be central to immune control of lung cancer via its effect on NK cell functional competency.

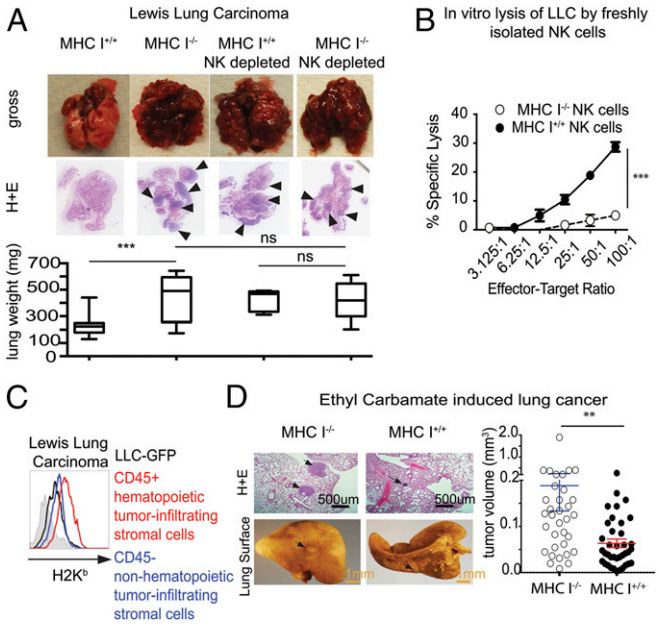

Fig. 1. NK cells from MHCI$^{+/+}$ mice offer superior control of lung cancer. (*A*) Growth of LLC in the lungs of mice 16 d after i.v. injection. The two panels on the *Left* represent IgG control-treated mice while the two panels on the *Right* represent similar tumor growth after NK cell depletion using anti-NK1.1 (clone PK136) before tumor injection. Representative of five to six animals per group. (*B*) In vitro lysis of LLC by freshly isolated NK cells from MHCI$^{+/+}$ or MHCI$^{-/-}$ mice. Representative of two separate assays containing five replicates per group. (*C*) MHCI expression of LLC-GFP in the flank (black line) in comparison with nonhematopoietic CD45− (blue) or CD45+ hematopoietic (red) stromal cells in the tumor microenvironment. (*D*) Ethyl carbamate (urethane) induced primary lung cancer burden in MHCI$^{-/-}$ and MHCI$^{+/+}$ mice evaluated by H&E (200×) (*Top Left*), gross inspection (*Bottom Left*), and direct tumor measurement (*Right*). ns, $P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Tumor transplant experiments consisted of 1×10^6 LLC injected s.c. for the flank tumor model and 1×10^5 injected i.v. for the lung tumor model.

Expression of NKG2D- and NKp46-Activating Receptors and Signaling Intermediates Within the PI3K-mTOR Pathway Is Modulated by MHCI Exposure. Previous reports have suggested that both NKG2D and NKp46 can recognize lung cancer (2, 5). Consistent with this, we were able to detect NKG2D and NKp46 stress ligands on LLC in vivo. Down-regulation of ligands during tumor progression in both MHCI$^{+/+}$ and MHCI$^{-/-}$ mice suggests a form of tumor editing (Fig. 2*A*) (17). While stress ligands recognized by other NK cell receptors, such as DNAM-1, were also detectable on LLC (*SI Appendix*, Fig. S2*A*), we focused heavily on the NKG2D- and NKp46-activating receptors due to their described role in multiple disease processes (18). LLC grew more rapidly in both NKG2D-KO and NKp46-KO mice in an NK cell-dependent fashion (Fig. 2*B*). NK cells from MHCI$^{+/+}$ mice were also more responsive to activation through NKG2D, NKp46, and DNAM in comparison with NK cells from MHCI$^{-/-}$ animals (Fig. 2*C*) (*SI Appendix*, Fig. S2*B*). The combination of NKG2D and NKp46 stimulation resulted in maximal degranulation and IFN-γ production, and the addition of DNAM stimulation did not increase activation any further. However, DNAM stimulation alone was as effective as NKp46 for NK cell degranulation and IFN-γ production (Fig. 2*C*) (*SI Appendix*, Fig. S2*B*). Such differences between MHCI$^{+/+}$ and MHCI$^{-/-}$ mice were not due to increased cytokine stimulation, as both strains of tumor-bearing mice had similar levels of systemic and lung IL2, IL15, and IL10 (*SI Appendix*, Fig. S2*C*). Taken together, these data imply that key activating receptors play a critical role in immunologic control of LLC growth by cell cells in MHCI$^{+/+}$ mice.

To explore this in greater detail, we performed detailed flow cytometric analysis of splenic NK cells from MHCI$^{-/-}$ and MHCI$^{+/+}$ mice. No differences were evident in the number or maturity state of NK cells between MHCI$^{-/-}$ and MHCI$^{+/+}$ mice (*SI Appendix*, Fig. S3 *A* and *B*). However, higher density of NKp46- and NKG2D-activating receptors was evident on the surface of MHCI$^{+/+}$ NK cells (Fig. 3*A*). Such differences were not evident for all receptors as NK1.1, Ly49H, 2B4, Ly49A, LyG2, and CD16/32 levels were similar between MHCI$^{+/+}$ and MHCI$^{-/-}$ NK cells, but others, such as DNAM and KLRG1, increased as well (Fig. 3*A*) (*SI Appendix*, Fig. S3*C*). A similar pattern of receptor expression was evident for lung-resident NK cells and NK cells from urethane-treated mice (*SI Appendix*, Fig. S3 *D* and *E*).

Most NK cell-activating receptors signal by association with an immunoreceptor-based activation motif containing adaptor proteins that activate the PI3k-AKT pathway (Fig. 3*B*). In previous work, Ardolino et al. (19) found that Erk and PI3K phosphoproteins were modified in NK cells in MHCI$^{+/+}$ mice by exposure to large numbers of transplanted MHCI-deficient RMA-S tumor cells. We thus examined proteins within the PI3k pathway by mass spectroscopy. To strengthen our analysis, we examined the proteomes obtained from two models of MHCI deficiency, namely β2-microglobulin$^{-/-}$ and triple knockout mice, with deletion of H2-K^b, D$^{b-/-}$, and β2-microglobulin (8). Proteomic analysis revealed a decrease in PI3K catalytic subunit delta and mTOR (Fig. 3*B*). Both of these intermediates are a part of the IL-15–mediated PI3K signaling pathway and lead to AKT phosphorylation (20). Consistent with decreased levels of these kinases, we found significantly lower levels of phosphorylated AKT in NK cells of MHCI$^{-/-}$ mice both before and after NKp46/NKG2D stimulation. The total AKT content of MHCI$^{+/+}$ and MHCI$^{-/-}$ NK cells was similar, supporting the notion that differences in phospho-AKT were the result of decreased upstream kinase activity (*SI Appendix*, Fig. S3*F*). Taken together, these data indicate that exposure to MHCI modulates levels of activating receptors and downstream signaling kinases that may alter NK activation.

Exposure to MHCI Reverses Defects in the NKp46 and NKG2D Pathways of NK Cell Activation and Decreases Lung Cancer Growth. Previous work has demonstrated that NK cell anergy due to development in

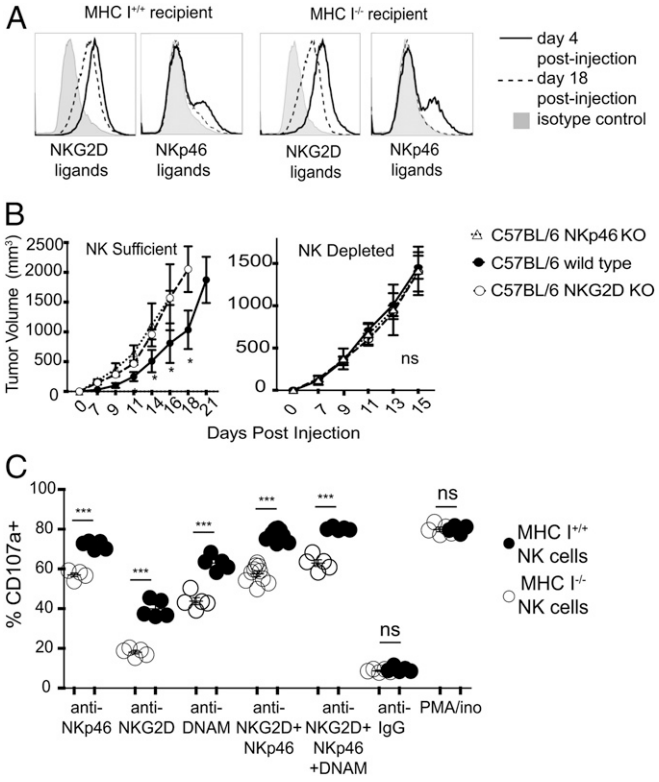

Fig. 2. NKG2D, NKp46, and DNAM reactivity of NK cells. (*A*) NKG2D and NKp46 ligand expression on progressively growing LLC-GFP. (*B*) Flank growth of LLC in wild-type C57BL/6, NKG2D-KO, and NKp46-KO mice. (*Left*) Tumor growth in IgG control-treated (NK cell-sufficient) and (*Right*) tumor growth in NK cell-depleted (PK136-treated) mice. To specifically focus on the role of NK cells, all mice were concurrently depleted of CD8+ T cells before tumor injection (clone YTS-169). Representative of four to five animals per group. (*C*) Degranulation as measured by surface CD107a after plate-bound stimulation with various activating antibodies or IgG isotype control in NK cells from either MHCI$^{-/-}$ or MHCI$^{+/+}$ mice. Stimulation performed overnight (15 h) in flat-bottom plates coated with 5 μg/mL of antibody for 3 h before addition of splenocytes. ns, $P > 0.05$; ***$P < 0.001$. Tumor transplant experiments consisted of 1×10^6 LLC or LLC-GFP injected s.c. for the flank tumor model.

MHCI$^{-/-}$ hosts is reversible upon transfer into MHCI$^{+/+}$ hosts. This effect has been referred to as re-education or "relicensing" (10, 12, 13). We next tested whether NK cell expression of NKp46 and NKG2D activation receptors, and downstream signaling components, are similarly subject to regulation by host MHCI expression. To accomplish this, we transferred MHCI$^{-/-}$ NK cells to MHCI$^{+/+}$ CD45 congenic hosts and compared their phenotype and function to unmanipulated MHCI$^{-/-}$ NK cells. Two weeks after transfer, an increase in surface NKG2D/NKp46, phosphorylated AKT, and IFN-γ production was evident in MHC$^{-/-}$ NK cells compared with pre-transfer levels (*SI Appendix*, Fig. S4*A*). Similarly, transfer of MHCI$^{+/+}$ NK cells into MHCI$^{-/-}$ hosts decreased surface expression of NKp46, NKG2D, and receptor-mediated production of IFN-γ (*SI Appendix*, Fig. S4*B*). By demonstrating that surface levels of both activating receptors and phosphorylated signaling intermediates increase upon exposure to MHCI, we now provide a mechanistic and physiologic explanation for the reversal of anergy or the "dysfunction" described for NK cells that reside in the MHCI$^{-/-}$ environment (10, 12).

Introduction of bone marrow-derived cells using non-myeloablative techniques has been proposed as a strategy to treat hematopoietic disorders (21). To explore the potential for "reactivating" NK cells in an MHCI$^{-/-}$ environment using a clinically relevant therapeutic strategy, we transferred MHCI$^{+/+}$ bone marrow to MHCI$^{-/-}$ mice utilizing a nonmyeloablative minimal conditioning regimen of 500 cGy irradiation (Fig. 4*A*). Using CD45 congenic donors, we were able to demonstrate that at 1 mo after transplantation, half of all hematopoietic bone marrow and spleen-resident cells were donor-derived and expressed H2K^b with multilineage chimerism (Fig. 4*A*) (*SI Appendix*, Fig. S4*C*). Recipient-derived MHCI$^{-/-}$ NK cells that developed in such a mixed environment were more responsive to NKp46/NKG2D stimulation than NK cells in a pure MHCI$^{-/-}$ environment (Fig. 4*A*). NK cell-mediated control of LLC growth was also more robust when MHCI$^{-/-}$ NK cells were exposed to such a mixed hematopoietic environment containing MHCI$^{+/+}$ antigen-presenting cells (Fig. 4*B*). Thus, even partial restoration of environmental MHCI can reverse the dysfunction of MHCI$^{-/-}$ NK cells.

Ly49C/I-Expressing NK Cells Play a Critical Role in Control of Lung Cancer. In C57BL/6 mice, Ly49C and -I represent the only Ly49 inhibitory receptors capable of binding MHCI (H2^b) (9). Other inhibitory receptors such as Ly49G2 and Ly49A, while expressed, are nonfunctional as their ligand, H2^d, is not present in the C57BL/6 strain (22). Based on the above data demonstrating the importance of MHCI, we thus assumed that the Ly49C/I$^+$ NK cells, educated or "licensed" by H2K^b, play a critical role in tumor control. We next depleted Ly49C/I$^+$ NK cells from MHCI$^{+/+}$ mice using the anti-Ly49C/I clone 5E6 before injection of LLC and noted that such treatment completely eliminated NK cell-mediated protection against lung cancer. In fact, mice depleted of Ly49C/I$^+$ cells demonstrated rapid tumor growth, similar in kinetics to mice depleted of all NK cells or MHCI$^{-/-}$ mice with unlicensed NK cells (Fig. 5*A*). Depletion of Ly49G2-expressing NK cells did not demonstrate such a dramatic effect on lung cancer growth (*SI Appendix*, Fig. S5*A*). Thus, the presence of licensed Ly49C/I$^+$ NK cells in MHCI$^{+/+}$ mice was required to effectively control LLC tumor progression.

To our surprise when we examined LLC-bearing tissues, we noted that LLC tumors were infiltrated by Ly49C/I$^+$ and Ly49C/I$^-$ NK cells that had degranulated, as measured by surface expression of CD107a (Fig. 5*B*). In fact, a relatively higher proportion of NK cells were Ly49C/I$^-$ in tumor-bearing tissue compared with the spleen (Fig. 5*B*). Such data suggested that both Ly49C/I$^+$ and Ly49C/I$^-$ NK cells may contribute to lung cancer-specific immune responses and seemed at odds with data demonstrating the seemingly critical role of the Ly49C/I$^+$ NK cells in tumor control.

NK Cell Activation Results in the Down-Regulation of Surface Ly49C/I Due to Receptor Shedding. To evaluate this apparent dichotomy, we stimulated MHCI$^{+/+}$ splenocytes with anti-NKp46 and NKG2D agonistic antibodies in vitro and noted degranulation by both Ly49C/I$^+$ and Ly49C/I$^-$ NK cells, as well as a decrease in the relative proportion of NK cells expressing Ly49C/I (Fig. 5*C*), similar to the in vivo data described above (Fig. 5*B*). This suggested a relative expansion of Ly49C/I$^-$ NK cells, contraction of the Ly49C/I$^+$ cell population, or down-regulation of Ly49C/I. A similar decrease in Ly49C/I$^+$ NK cells was evident after stimulation of other activating receptors, such as NK1.1 and Ly49H (*SI Appendix*, Fig. S5*B*). Interestingly levels of NKG2A, another inhibitory receptor capable of ligand interaction in C57BL/6 mice, or of Ly49G2 or Ly49A, did not vary after NK cell stimulation. Such a decrease in Ly49C/I, but not in Ly49G2 or Ly49A, was also evident in BALB/c NK cells after plate-bound stimulation with anti-NKp46 and anti-NKG2D (*SI Appendix*, Fig. S5 *B* and *C*).

Based on the dynamic regulation of NKG2D and NKp46, we next decided to evaluate whether surface expression of Ly49C/I also varied based on environmental context. We thus adoptively transferred flow cytometrically sorted Ly49C/I$^+$ CD45.1$^+$ congenic NK cells into CD45.2$^+$ mice bearing LLC and evaluated surface expression of Ly49C/I 15 h later. We noted down-regulation of Ly49C/I in a significant portion of the previously Ly49C/I$^+$ cells in tumor-bearing lungs (Fig. 5*D*). Ly49C/I levels

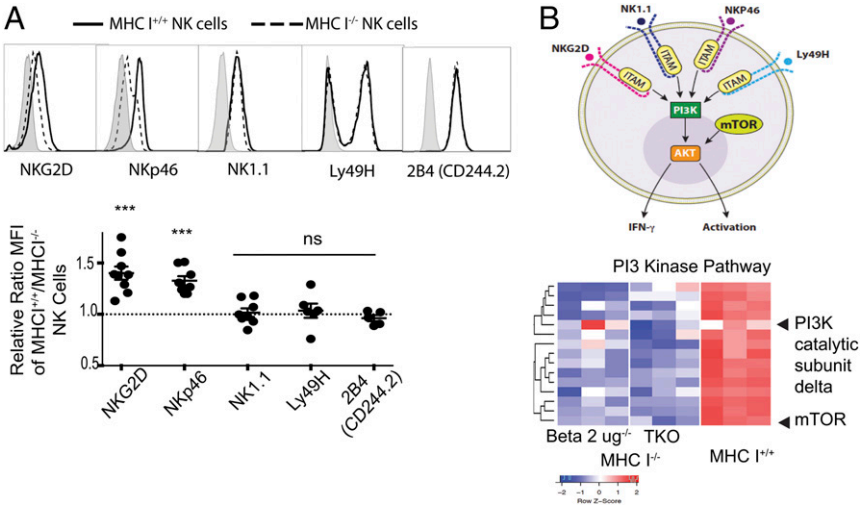

Fig. 3. NK cell phenotype. (*A*) Representative histogram (*Top*) and relative ratio of surface median fluorescence intensity (MFI) of various activating receptors in NK cells from MHCI$^{+/+}$/MHCI$^{-/-}$ mice (*Bottom*). (*B*) Graphic representation of signaling pathways of NK cell-activating receptors (*Top*). Heat map representation of mass spectroscopy of a portion of the PI3K-signaling pathway (*Bottom*). ns, $P > 0.05$; ***$P < 0.001$.

remained high in nontumor-bearing tissues such as the spleen. Similar to in vivo data, in vitro activation of sorted Ly49C/I$^+$ NK cells resulted in the down-regulation of these inhibitory receptors on the surface of NK cells as well (Fig. 5*D*). In direct contrast, stimulation of NK cells resulted in up-regulation of the activating receptor NKG2D (*SI Appendix*, Fig. S5*D*).

To evaluate the mechanism/s responsible for the decrease in surface inhibitory receptor expression, we next quantified mRNA and total protein levels of Ly49C and -I from sorted Ly49C/I$^+$ NK cells. Increased levels of both Ly49C/I mRNA (*SI Appendix*, Fig. S5*E*) and total protein levels, as determined by Western blotting, were evident in stimulated NK cells (Fig. 5*E*). Thus, the decrease in surface expression is not the result of decreased protein synthesis. To evaluate if decreased surface levels were due to increased internalization, we next stimulated sorted Ly49C/I$^+$ NK cells in cultures containing fluorescein isothiocyanate (FITC)-conjugated anti-Ly49C/I antibody with the addition of monensin to prevent fluorochrome degradation upon receptor internalization. By costaining for surface NK1.1, we were able to detect Ly49C/I that had internalized during the course of stimulation as opposed to that located on the surface of the cell. No differences were detected in Ly49C/I internalization between anti-NKG2D/NKp46–activated and unstimulated NK cells (Fig. 5*F*).

Previous investigators have implicated the multifunctional β-arrestin in the homeostasis of NK cell inhibitory receptors such as Ly49C (23). We next explored whether activation-induced changes in β-arrestin might be responsible for changes in Ly49C/I levels but were unable to detect differences in this protein upon activation (*SI Appendix*, Fig. S5*F*). It has been described that NK cells can modulate their functional capacity through shedding of various receptors such as CD16 (24). To evaluate if shedding could be responsible for the down-regulation of surface inhibitory receptors, we compared levels of free Ly49C in media from resting or NKG2D/NKp46-stimulated NK cell cultures by ELISA. Higher levels of shed, non-NK-cell–bound Ly49C were evident after activation (Fig. 5*G*), suggesting that receptor shedding plays a role in the down-regulation of surface expression.

Discussion

While NK cells licensed or educated by environmental MHCI readily lyse MHCI$^{-/-}$ targets, inhibitory receptor-mediated target interference may interfere with cytotoxicity toward MHCI$^{+/+}$ targets. For these reasons, inhibitory ligand–MHCI mismatching has been utilized for antileukemia effects of allogeneic bone marrow transplantation (BMT), with increasing efficacy at the highest number of mismatches (25). Clearance of certain viral infections (26) and ADCC-mediated control of neuroblastoma (27) have been reported to be augmented by NK cells missing their respective inhibitory ligand(s) due to lack of target inhibition upon pathogen encounter (27).

Here we demonstrate that NK cells of C57BL/6 (H2K^b) mice licensed by MHCI through Ly49C/I provide a substantial barrier to the growth of lung cancer. We also demonstrate that surface

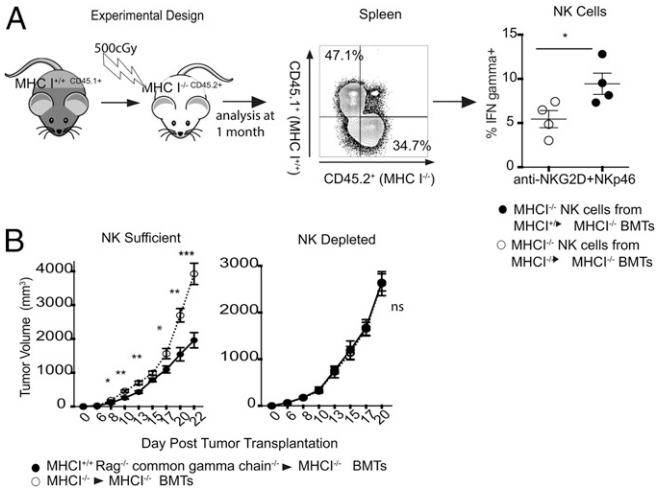

Fig. 4. Exposure to MHCI alters NK cell reactivity. (*A*) Graphic representation of nonmyeloablative BMTs (*Left*) with demonstration of mixed chimerism in the spleen (*Middle*) and reactivity of MHC$^{-/-}$ CD45.2+ recipient NK cells after plate-bound stimulation by NKG2D and NKp46 agonistic antibodies (*Right*). Stimulation performed overnight (15 h) in flat-bottom plates coated with 5 μg/mL of antibody for 3 h before addition of splenocytes. (*B*) Growth of LLC injected into the flank of MHCI$^{-/-}$ to MHCI$^{-/-}$ or MHCI$^{+/+}$ to MHCI$^{-/-}$ nonmyeloablative BMTs. To prevent the effect of donor-derived NK cells, Rag$^{-/-}$common gamma chain$^{-/-}$ mice were used as source of MHCI$^{+/+}$ bone marrow. Representative of isotype control-treated mice (*Left*) or those depleted of NK cells by anti-NK1.1 monoclonal antibody (clone PK136) (*Right*). Graph representative of five mice per group. ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Tumor transplant experiments consisted of 1×10^6 LLC injected s.c. for the flank tumor model.

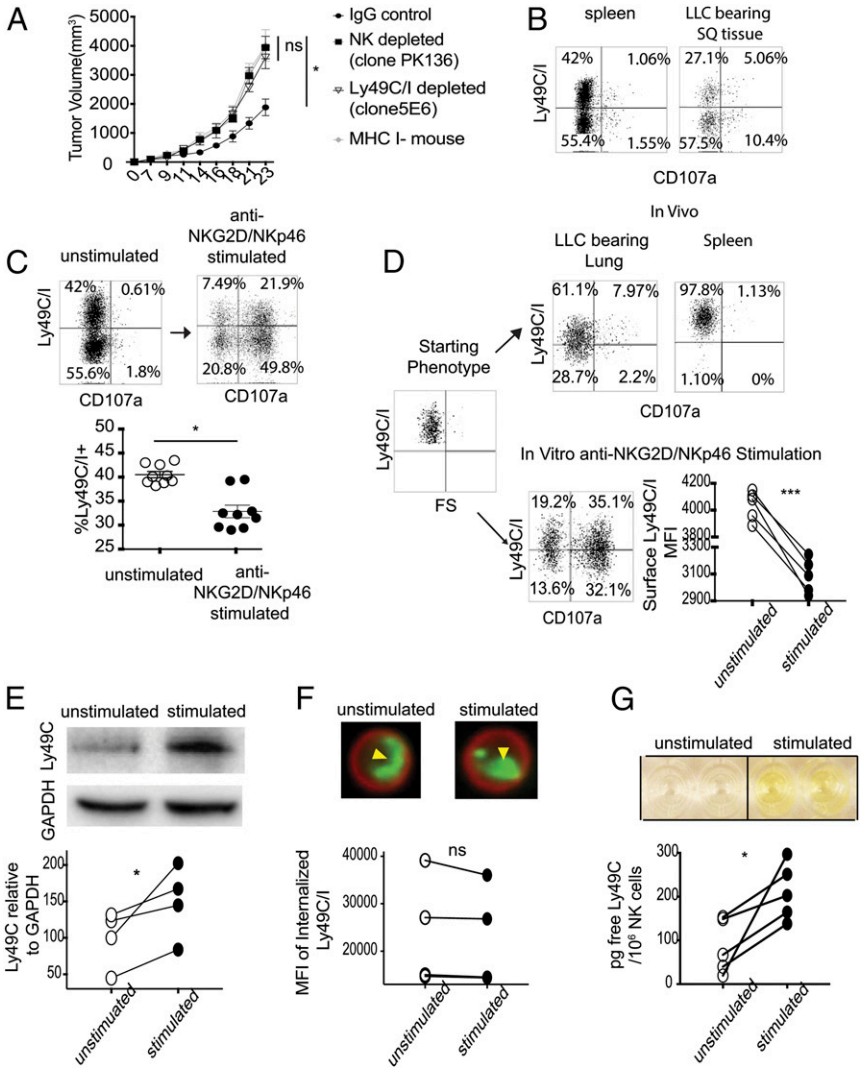

Fig. 5. Dynamic regulation of Ly49C/I inhibitory receptor expression. (*A*) LLC growth in MHCI$^{+/+}$ mice depleted of Ly49C/I^{+} NK cells or all NK cells compared with IgG isotype control-treated or MHCI$^{-/-}$ mice. Representative of five mice per group. (*B*) Ly49C/I expression and degranulation (as measured by CD107a expression) in splenic NK cells or NK cells infiltrating s.c. (SQ) LLC nodules. (*C*) Ly49C/I expression and degranulation in resting and anti-NKG2D/NKp46–activated NK cells. (*Top*) Representative plots. (*Bottom*) Data summary. (*D*) Ly49C/I expression in sorted CD45.1+ congenic NK cells adoptively transferred into LLC-bearing CD45.2+ congenic mice (*Top*) or after in vitro stimulation with anti-NKG2D/NKp46 in vitro (*Bottom*). Representative of three separate experiments in vivo. (*E*) Total Ly49C levels in freshly isolated NK cells cultured with plate-bound IgG control (unstimulated) or anti-NKG2D/NKp46 (stimulated) as determined by Western blotting. (*Top*) Representative blot. (*Bottom*) Graphic representation. (*F*) Image stream quantification of internalized Ly49C/I as determined by FITC fluorescence (yellow arrowheads). (*G*) Free shed Ly49C in serum of freshly isolated NK cells cultured with plate-bound IgG control (unstimulated) or anti-NKG2D/NKp46 (stimulated) as determined by indirect ELISA. (*Top*) Representative ELISA wells. (*Bottom*) Quantification based on Ly49C standard curve. ns, $P > 0.05$; $^{*}P < 0.05$; $^{***}P < 0.001$. Tumor transplant experiments consisted of 1×10^6 LLC injected s.c. for the flank tumor model and 1×10^5 injected i.v. for the lung tumor model. Stimulation was performed overnight (15 h) in flat-bottom plates coated with 5 μg/mL of antibody for 3 h before addition of splenocytes.

expression of the inhibitory receptor Ly49C/I, which has a high affinity for H2K^{b} (22), is down-regulated upon NK cell activation through the process of receptor shedding. To our knowledge, such shedding of inhibitory receptors has not been previously described but physiologically may resemble homeostasis of CD16, where shedding from the surface of NK cells has been described to facilitate motility, target detachment, and serial killing (24). It is possible that a similar system of shedding may allow for NK cell licensing while at the same time ameliorating target interference upon activation. We found it interesting that other inhibitory receptors, such as Ly49G2 and Ly49A, are not down-regulated. It is possible that Ly49C, with its ability to broadly bind multiple MHCI alleles with high affinity (22), is regulated differently from other less promiscuous inhibitory receptors with the physiologic details of such a system yet to be defined.

For the purposes of our study, we varied the duration of stimulation from the traditional 4- to 6-h period. By relying on a longer 15-h stimulation in vitro, we model in vivo adoptive transfer of NK cells (Fig. 5*D*). Such a protocol may also mirror the prolonged duration of receptor engagement that an NK cell may have through multiple encounters with serial targets in vivo (28). For the purpose of this study, we focused on the Ly49C/I inhibitory receptor due to its high affinity interaction with H2K^{b} and other MHC class I alleles (22). We do recognize that other inhibitory receptors, such as CD94/NKG2A, may contribute to NK cell licensing or education in C57BL/6 mice (9) but, as described above, levels of NKG2A did not vary upon stimulation (*SI Appendix*, Fig. S5*B*). These data further support the importance of Ly49C/I in the homeostasis of murine NK cells due to its high affinity for MHCI (22) and put into question the strict use of NK cell inhibitory receptor expression, evaluated at a single time point, to define the licensing status of an NK cell. We also recognize that, due to a limited number of tumor-infiltrating NK cells, functional assays could not be done with this cell population. Thus, we had to rely on phenotypic analysis of NK cells within the tumor bed while performing functional assays with spleen-derived cells.

Previous investigators have defined functional differences in inhibitory ligand-licensed NK cells but describe limited phenotypic differences to account for such variability (11, 19, 29, 30). Using a broad-based platform, we identify a select set of receptors (NKG2D, NKp46, DNAM-1, and KLRG1), the surface expression of which is altered upon exposure to MHCI. We further identify components within the PI3K-mTOR-AKT pathway that are similarly affected by MHCI-mediated licensing. It is interesting that, while NKp46, NKG2D, and DNAM belong to different and genetically unrelated families of activating receptors, all are down-regulated in an MHCI$^{-/-}$ environment while surface expression of other activating receptors, such as Ly49H or NK1.1, remains similar. Ligands recognized by NKp46- and NKG2D-activating receptors are expressed on stressed or dying cells (31, 32) while

the DNAM ligands CD155 (poliovirus receptor) and CD112 (nectin-2) are expressed on a broad range of cells at baseline and upon transformation (33). It is thus possible that these pathways are continuously activated as part of normal tissue senescence or cellular turnover. NK cells subject to such chronic and unopposed stimulation may thus undergo compensatory down-regulation of surface-activating receptors and downstream-signaling intermediates, similar to that described for T lymphocytes (34). Such down-regulation may be more pronounced in an MHCI-deficient environment where inhibitory ligand engagement does not occur. Activating receptors such as NK1.1 or Ly49H, which have no known ligands in resting mice, may not be subject to chronic ongoing stimulation and down-regulation. This notion is supported by the "disarming" theory of NK cell education, which links hyporesponsiveness of NK cells deficient in inhibitory receptors to chronic unopposed activation (30). A recent report that partial blockade of NKG2D by soluble MULT1 may actually augment cytotoxicity of this pathway further supports this concept (35).

Our data demonstrate that NK cell cytotoxicity, and its control of cancer growth, can be improved through the introduction of the cognate MHCI antigen as part of minimally myeloablative chimerism. Such data extend clinical applications of our work since it is conceivable to introduce MHCI alleles missing for inherited inhibitory ligands through allogenic bone marrow transplantation. Such data also support the previously proposed notion that the expression of MHCI solely on antigen-presenting cells in the environment is sufficient to alter NK cell function and relicense NK cells (10). Thus, cis-interactions, or the engagement of inhibitory ligands and MHCI on the same cell, are not an absolute prerequisite to NK cell licensing (36). Our data pave the way for relicensing or re-education of NK cells as part of multimodality immunotherapy for lung cancer (1, 37, 38).

Materials and Methods

Detailed materials and methods are presented fully in *SI Appendix, SI Materials and Methods*. All mice used in the study were generated on a C57BL/6 background and were either purchased from Jackson Laboratories or obtained as a gift from Ted Hanson, Washington University, St. Louis (*SI Appendix*, Table S1). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Virginia. All antibodies for cell depletion were purchased from BioXcell, and all isolation kits were purchased from Miltenyi Biotec. Flow cytometry staining was performed on ice in staining buffer (2% FCS, 0.1% NaN3 in PBS) containing anti-FcR antibodies (2.4G2). The full list of fluorochrome-conjugated antibodies is presented in *SI Appendix, SI Materials and Methods*. For plate-bound stimulation, anti-NKP46 (29A1.4) and anti-NKG2D (A10) antibodies at 5 μg/mL were bound on plastic (24-well) plates for 3 h in PBS, washed twice with PBS, and cultured overnight with NK cells or splenocytes as described throughout the paper. Quantification of Ly49C shedding from NK cells was performed by indirect ELISA with Ly49C and protein concentration calculated based on a standard curve of an *Escherichia coli*-expressed protein refolded as previously described (39).

ACKNOWLEDGMENTS. A.S.K. was supported by Grants PO1AI116501 (Washington University in St. Louis), 1I01BX002299 (Salem VA Medical Center), and R41CA224520 (University of Virginia), and work in the manuscript was partially supported by the University of Virginia (UVA) Cancer Center National Cancer Institute Grant P30-CA044579-23 and UVA Cancer Center Bioinformatics Core via Grant P30CA044579.

1. Guillerey C, Huntington ND, Smyth MJ (2016) Targeting natural killer cells in cancer immunotherapy. *Nat Immunol* 17:1025–1036.
2. Kreisel D, et al. (2012) Strain-specific variation in murine natural killer gene complex contributes to differences in immunosurveillance for urethane-induced lung cancer. *Cancer Res* 72:4311–4317.
3. Frese-Schaper M, et al. (2014) Influence of natural killer cells and perforin-mediated cytolysis on the development of chemically induced lung cancer in A/J mice. *Cancer Immunol Immunother* 63:571–580.
4. Arefanian S, et al. (2016) Deficiency of the adaptor protein SLy1 results in a natural killer cell ribosomopathy affecting tumor clearance. *OncoImmunology* 5:e1238543.
5. Glasner A, et al. (2012) Recognition and prevention of tumor metastasis by the NK receptor NKp46/NCR1. *J Immunol* 188:2509–2515.
6. Malarkannan S (2006) The balancing act: Inhibitory Ly49 regulate NKG2D-mediated NK cell functions. *Semin Immunol* 18:186–192.
7. He Y, Tian Z (2017) NK cell education via nonclassical MHC and non-MHC ligands. *Cell Mol Immunol* 14:321–330.
8. Kim S, et al. (2005) Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436:709–713.
9. Fernandez NC, et al. (2005) A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 105: 4416–4423.
10. Elliott JM, Wahle JA, Yokoyama WM (2010) MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J Exp Med* 207:2073–2079.
11. Ebihara T, Jonsson AH, Yokoyama WM (2013) Natural killer cell licensing in mice with inducible expression of MHC class I. *Proc Natl Acad Sci USA* 110:E4232–E4237.
12. Joncker NT, Shifrin N, Delebecque F, Raulet DH (2010) Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *J Exp Med* 207: 2065–2072.
13. Wagner AK, et al. (2016) Retuning of mouse NK cells after interference with MHC class I sensing adjusts self-tolerance but preserves anticancer response. *Cancer Immunol Res* 4:113–123.
14. Koller BH, Marrack P, Kappler JW, Smithies O (1990) Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. *Science* 248:1227–1230.
15. Fung-Leung WP, et al. (1991) CD8 is needed for development of cytotoxic T cells but not helper T cells. *Cell* 65:443–449.
16. Smyth MJ, Crowe NY, Godfrey DI (2001) NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol* 13:459–463.
17. O'Sullivan T, et al. (2012) Cancer immunoediting by the innate immune system in the absence of adaptive immunity. *J Exp Med* 209:1869–1882.
18. Lünemann A, et al. (2008) Human NK cells kill resting but not activated microglia via NKG2D- and NKp46-mediated recognition. *J Immunol* 181:6170–6177.
19. Ardolino M, et al. (2014) Cytokine therapy reverses NK cell anergy in MHC-deficient tumors. *J Clin Invest* 124:4781–4794.
20. Nandagopal N, Ali AK, Komal AK, Lee SH (2014) The critical role of IL-15-PI3K-mTOR pathway in natural killer cell effector functions. *Front Immunol* 5:187.
21. Gyurkocza B, et al. (2010) Nonmyeloablative allogeneic hematopoietic cell transplantation in patients with acute myeloid leukemia. *J Clin Oncol* 28:2859–2867.
22. Hanke T, et al. (1999) Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity* 11:67–77.
23. Yu MC, et al. (2008) An essential function for beta-arrestin 2 in the inhibitory signaling of natural killer cells. *Nat Immunol* 9:898–907.
24. Srpan K, et al. (2018) Shedding of CD16 disassembles the NK cell immune synapse and boosts serial engagement of target cells. *J Cell Biol* 217:3267–3283.
25. Leung W, et al. (2004) Determinants of antileukemia effects of allogeneic NK cells. *J Immunol* 172:644–650.
26. Orr MT, Murphy WJ, Lanier LL (2010) 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat Immunol* 11:321–327.
27. Tarek N, et al. (2012) Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment. *J Clin Invest* 122:3260–3270.
28. Bhat R, Watzl C (2007) Serial killing of tumor cells by human natural killer cells: Enhancement by therapeutic antibodies. *PLoS One* 2:e326.
29. Andrews DM, et al. (2012) Recognition of the nonclassical MHC class I molecule H2-M3 by the receptor Ly49A regulates the licensing and activation of NK cells. *Nat Immunol* 13:1171–1177.
30. Raulet DH, Vance RE (2006) Self-tolerance of natural killer cells. *Nat Rev Immunol* 6: 520–531.
31. Groh V, et al. (1996) Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci USA* 93: 12445–12450.
32. Bloushtain N, et al. (2004) Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. *J Immunol* 173: 2392–2401.
33. Zhang Z, et al. (2015) DNAM-1 controls NK cell activation via an ITT-like motif. *J Exp Med* 212:2165–2182.
34. Schönrich G, et al. (1991) Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell* 65:293–304.
35. Deng W, et al. (2015) Antitumor immunity. A shed NKG2D ligand that promotes natural killer cell activation and tumor rejection. *Science* 348:136–139.
36. Chalifour A, et al. (2009) A role for cis interaction between the inhibitory Ly49A receptor and MHC class I for natural killer cell education. *Immunity* 30:337–347.
37. Gras Navarro A, Björklund AT, Chekenya M (2015) Therapeutic potential and challenges of natural killer cells in treatment of solid tumors. *Front Immunol* 6:202.
38. Stojanovic A, Correia MP, Cerwenka A (2013) Shaping of NK cell responses by the tumor microenvironment. *Cancer Microenviron* 6:135–146.
39. Sullivan LC, et al. (2016) Recognition of the major histocompatibility complex (MHC) class Ib molecule H2-Q10 by the natural killer cell receptor Ly49C. *J Biol Chem* 291: 18740–18752.